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Botulinum Neurotoxins Are Zinc Proteins*

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The available amino acid sequences of 150-kDa botulinum and tetanus neurotoxins show the presence of a closely homologous segment in the middle of the light chain (NH_2-terminal 50 kDa), which is the intracellularly active portion of the toxin. This segment contains the zinc binding motif of metalloendopeptidases, HEXXH. Atomic adsorption analysis of botulinum neurotoxins (serotypes A, B, and E) made on the basis of this observation demonstrated the presence of one zinc atom/molecule of 150-kDa neurotoxin. Conditions were found for the removal of the zinc ion with chelating agents and for the restoration of the normal metal content. The conserved segment, which includes the zinc binding motif, was synthesized and shown to bind $[^{65}Zn]^{2+}$.

Chemical modification experiments indicated that two histidines and no cysteines are involved in Zn^{2+} coordination in agreement with a probable catalytic role for the zinc ion. The present findings suggest the possibility that botulinum neurotoxins are zinc proteases.

Botulinum neurotoxins (BoNT)[1] are produced in seven different serotypes (A, B, C1, D, E, F, and G) by *Clostridium botulinum* and by other species of the same genus (Simpson, 1989; Hatheway, 1990). They are the most potent bacterial protein toxins. All the clinical symptoms of botulism, including the flaccid paralysis, are due to BoNT, which blocks acetylcholine release at the neuromuscular junction. Their enormous potency has been attributed to their neurospecificity and to a yet unknown intracellular enzymic activity as it is the case for all bacterial protein toxins with cytosolic targets (Simpson, 1989; Alouf and Freer, 1991).

BoNTs are produced as 150-kDa single polypeptide chains. Proteolytic cleavage within a narrow region generates a two-chain form of BoNT composed of an H chain (100 kDa) and an L chain (50 kDa) held together by a disulfide bridge and noncovalent bonds (Fig. 1). The two-chain form is generally more active than the single chain (DasGupta, 1989). The H chain is responsible for the neurospecific binding of BoNT to peripheral nerve cells and presumably also for the cytosolic translocation of the L chain (Montecucco, 1986; Niemann, 1991). The L chain is the intracellularly active portion of the neurotoxin that blocks neuroexocytosis (Poulain *et al.*, 1988, 1990, 1991). However, despite all efforts, neither its intracellular mode of action nor its target is yet known.

We suggest here the possibility that BoNTs are metalloendopeptidases. This suggestion is based on the following findings. All clostridial neurotoxins, whose sequences are available, contain the zinc binding motif of zinc endopeptidases. Consequently, we investigated the possibility that BoNTs are metalloproteins by measuring the metal content of serotypes A, B, and E. These three serotypes were chosen because they are most frequently involved in human botulism (Hatheway, 1990) and are available as highly pure preparations. We found that indeed one atom of zinc is bound per molecule of BoNT, this binding is reversible, and histidines are involved in zinc coordination, as in all zinc endopeptidases.

MATERIALS AND METHODS

Purification of Neurotoxins and Related Fragments

BoNT serotypes A, B, and E were produced and purified as previously described (DasGupta and Rasmussen, 1983; DasGupta and Sathyamoorthy, 1984; DasGupta and Woody, 1984). Serotypes A and E were in two-chain and single chain forms, whereas serotype B was essentially single chain. BoNT/B and BoNT/E were nicked with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Serva) as described by Sathyamoorthy and DasGupta (1985), and the cleavage was blocked by adding a 4-fold excess of soybean trypsin inhibitor.

The H and L chains of serotype A were separated and purified as reported previously (Sathyamoorthy and DasGupta, 1985). The 50-kDa carboxyl-terminal half of the H chain (H_C) and BoNT serotype A and the remaining 100-kDa fragment (L-H_N, the L chain, and the 50-kDa NH_2-terminal half of the H chain) were isolated following a procedure developed by Gimenez and DasGupta.[2] 5.0 mg of BoNT serotype A diluted to 0.5 mg/ml in 50 mM ammonium acetate, pH 4.1, was digested with pepsin (1:30, w/w) for 30 min at 35 °C. The reaction was blocked by adding 2 M Tris chloride, pH 8.8 (final concentration, 100 mM), and 1 μg/ml pepstatin. The reaction mixture was dialyzed extensively against 20 mM sodium phosphate, pH 8.0, and applied to a DEAE-Sephadex A-50 column 1.5 × 6.0 cm) equilibrated in the same buffer. H_C was recovered in the void volume, and the L-H_N fragment was eluted with a linear gradient of increasing sodium chloride; both were precipitated by ammonium sulfate (39 g/100 ml). Concentrations of H_C and L-H_N were determined from the absorbance at 278 nm using extinction coefficients of 1.7 and 1.27 M^{-1} cm^{-1}, respectively.

Diphtheria toxin (DT) was prepared as described (Rappuoli *et al.*, 1983).

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[1] The abbreviations used are: BoNT, botulinum neurotoxin; DEPC, diethyl pyrocarbonate; DT, diphtheria toxin.

[2] A. Gimenez and B. R. DasGupta, manuscript in preparation.

Determination of Metal Content

Each material (glassware, dialysis bags, etc.) used in metal determination experiments was previously rinsed with Milli-Q grade water (conductivity > 10 megaohms). Buffers were prepared with chemicals of the highest purity available with respect to the presence of heavy metals and pretreated with Amberlite MB-3 (Sigma). Before metal determination, the neurotoxins and the fragments thereof were extensively dialyzed at 4 °C against 150 mM Tris chloride, pH 7.4, or 10 mM HEPES-Na, 100 mM sodium chloride, pH 7.0. Protein samples and dialysis buffer were analyzed for zinc, cobalt, copper, iron, manganese, and nickel with a Perkin-Elmer 4000 atomic absorption flame spectrophotometer with impact bed loading after standardization for each ion in the linear range of concentration (0–0.5 ppm for zinc, nickel, and cobalt; 0–1 ppm for copper; 0–5 ppm for iron and manganese).

Zinc Removal and Reuptake

BoNT serotypes A, B, and E were diluted to 0.5–1.0 mg/ml with 150 mM Tris chloride, pH 7.4, and incubated in the presence of 10 mM Na-EDTA for 60 min at 37 °C. Samples were dialyzed extensively against the same buffer without EDTA at 4 °C before zinc content was determined. Zinc reuptake was accomplished by adding 100 μM zinc chloride dissolved in 150 mM Tris chloride, pH 7.4, to Zn^{2+}-depleted BoNT. After 60 min at 37 °C, the samples were extensively dialyzed against the same buffer without Zn^{2+} at 4 °C, and metal content was determined.

Diethyl Pyrocarbonate Modification

Native and zinc-depleted BoNT serotypes A, B, and E, between 1.5 and 2.5 μM in 50 mM sodium phosphate, pH 7.8, after filtration through a 0.22-μm filter (Anotec, Oxford, United Kingdom), were treated with DEPC in three consecutive additions of a 35-fold molar excess of reagent with respect to the toxin. DEPC was taken from a freshly prepared solution in absolute ethanol. The reaction was carried out at 25 °C and was monitored by simultaneous recording of the differential absorbances at 243 and 278 nm as previously described (Miles, 1977; Papini *et al.*, 1989) in a Perkin-Elmer Lamda 5 spectrophotometer. Modifications of histidine and tyrosine residues were estimated based on a differential extinction coefficient of 3,200 M^{-1} cm^{-1} at 243 nm for *N*-carbethoxyhistidine and of −1,310 M^{-1} cm^{-1} at 278 nm for *O*-carbethoxytyrosine.

Titration of Free Thiol Groups

Native or zinc-depleted BoNT serotypes A, B, and E, 1 μM in deaerated 50 mM sodium phosphate buffer, pH 7.8, were incubated with 0.4 mM 5,5′-dithiobis-(2-nitrobenzoic acid) at 25 °C. The absorbance at 410 nm was determined against a blank without neurotoxin. Based on a molar absorptivity of 13,600 M^{-1} cm^{-1} at 412 nm for the 2-nitro-5-thiobenzoate anion, sulfhydryl content was calculated as moles of free thiol groups/mol of BoNT (Ellman, 1959; Schiavo *et al.*, 1990).

Peptide Synthesis

The segment of BoNT serotype B, which spans 15 residues (226–240, H_2N-Ile-Leu-Met-His-Glu-Leu-Ile-His-Val-Leu-His-Gly-Leu-Tyr-Gly-COOH) was prepared by solid phase synthesis with a SMPS 350 automatic synthesizer (Zynsser Analytic, Frankfurt, Germany) employing Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry. The product was detached from the resin with 93% trifluoroacetic acid purified by high pressure liquid chromatography on a Vydac C18 column (Vydac, CA), and its amino acid sequence was verified by automatic Edman degradation on an Applied Biosystems microsequenator (model 475A).

Determination of $[^{65}Zn]^{2+}$ Binding

Zinc Overlay—0.6–20 pmol of BoNT serotypes A and E in their native and zinc-depleted forms, before and after DEPC treatment (as mentioned above), as well as 20–600 pmol of the synthetic peptide, were dot blotted onto nitrocellulose paper stripes (porosity, 0.22 μm) (Hoefer, CA). The same amount of diphtheria toxin was dot blotted as the control. The stripes were rinsed for 5 min in 25 mM Tris chloride, pH 7.5, containing 100 mM sodium chloride and then incubated for 60 min at room temperature in the presence of 50 nM $^{65}ZnCl_2$ (Amersham, U.K.) (specific activity, 575 mCi/mg zinc) in the same buffer. Unbound $^{65}ZnCl_2$ was removed by washing the stripes six times with 25 mM Tris chloride, 100 mM sodium chloride, pH 7.5. The amount of bound $[^{65}Zn]^{2+}$ was determined by exposing the dried stripes to Kodak X-Omat films at −80 °C.

Gel Filtration Chromatography—0.5 nmol of BoNT serotype E or 1.2 nmol of DT were incubated with 1.15 μCi of $^{65}ZnCl_2$ in 150 μl of 100 mM Tris chloride, 50 mM sodium chloride, pH 7.0, for 60 min at 25 °C and then were applied onto a Sephadex G-25 medium column (Pharmacia LKB Biotechnology Inc.) (100 × 8 mm), equilibrated, and eluted with the same buffer. Void volume (4.0 ml) was determined with blue dextran (Pharmacia). 0.9-ml fractions were collected, protein elution was monitored by UV absorption at 280 nm, and radioactivity was measured by counting in a Packard Multi-Prias Gamma counter. 0.5 μCi of $[^{65}Zn]^{2+}$ coeluted with the protein peak of BoNT, whereas background amounts were found in the DT peak.

Flow Dialysis

Binding of $[^{65}Zn]^{2+}$ to the BoNTs was measured by the flow dialysis technique of Colowick and Womack (1969). The apparatus consisted of two cylindric chambers (10 mm diameter; volumes: upper chamber, 1 ml; lower chamber, 0.18 ml) separated by a dialysis membrane. The toxins were preincubated for 45 h at 4 °C with 0.3 μM $[^{65}Zn]^{2+}$. The upper chamber routinely contained 1.7–2.7 μM BoNT and 0.3 μM $[^{65}Zn]^{2+}$ in a 400-μl volume of 20 mM Tris-Cl, 120 mM NaCl, 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.45, at room temperature. The lower chamber was perfused (0.5 ml/min) with the same buffer without zinc, and 0.5-ml fractions were collected and counted in a Packard Cobra Autogamma 5003 counter. 1–2-μl aliquots of $ZnCl_2$ were added to the upper chamber every 6 fractions.

Toxicity Tests

The neuroparalytic activity of BoNTs was tested by intravenous injection into BALB/c mice as described by Boroff and Fleck (1966).

RESULTS AND DISCUSSION

Sequence Comparison—The cDNA-derived amino acid sequences of BoNT serotypes A, B, C1, D, and E and from *Clostridium butyricum* as well as that of tetanus neurotoxin are known (Eisel *et al.*, 1986; Fairweather and Lyness, 1986; Hauser *et al.*, 1990; Binz *et al.*, 1990a, 1990b; Niemann, 1991; Thompson *et al.*, 1990; Poulet *et al.*, 1992). They show an overall low degree of homology with a few segments of close similarity. The most conserved segment among these clostridial neurotoxins, located in the central part of the light chain, is reported in the lower part of Fig. 1. The figure also shows that this segment includes the zinc binding motif of zinc endopeptidases (Vallee and Auld, 1990a).

Metal Content of Botulinum Neurotoxins—The above observation suggested to us the possibility that also the BoNTs are zinc proteins. To determine the nature and amount of metals bound to BoNT, highly purified preparations of BoNT serotypes A, B, and E, each in the two-chain form (their SDS-polyacrylamide gel electrophoresis profiles are in Fig. 2*A*), were subjected to atomic adsorption analysis. Fig. 2*B* shows that all three BoNT serotypes contain approximately 1 atom of zinc/molecule of 150-kDa toxin. Nicking of the BoNT serotypes B and E did not modify significantly the metal content (not shown). Analysis of the two peptic fragments of BoNT serotype A showed that the zinc atom is bound to the 100-kDa L-H_N fragment. The zinc content of the 50-kDa carboxyl-terminal fragment H_C was below detection limit. It was not possible to determine the metal content of the isolated 50-kDa L chains because separation of L and H chains required urea and dithiothreitol (Sathyamoorthy and DasGupta, 1985), which released the metal from the protein (not shown). Cobalt, copper, iron, manganese, and nickel were measured and found to be below detection limits.

Results presented in Fig. 2*B* also show that the Zn^{2+} atom was removed by treatment with EDTA. The loss of Zn^{2+} caused by EDTA was reversible because the zinc ion was reacquired by BoNT upon incubation in a Zn^{2+}-containing medium. On the contrary, Zn^{2+} reuptake by purified L chain

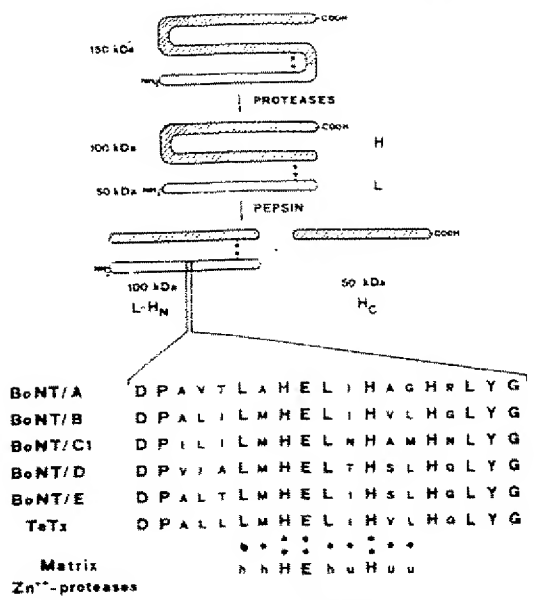

FIG. 1. **Schematic structure of clostridial neurotoxins and sequence comparison of their histidine-rich segment with the zinc binding motif of metalloendopeptidases.** Clostridial neurotoxins are produced as a single 150-kDa chain which is later cleaved by proteases at an exposed loop to generate the active two-chain toxin. The H chain (*H*) can be further cleaved with pepsin generating the 50-kDa H_C fragment. The most conserved segment of the sequences of BoNT A, B, C, D, and E and of tetanus toxin (*TeTx*), located in the central part of the L chain (*L*), is aligned, and it is shown to contain the Zn^{2+} binding motif of matrix metalloproteases: *h*, hydrophobic; *u*, uncharged. *Double dots* indicate residue conservation and *single dots* indicate conservation of residue properties.

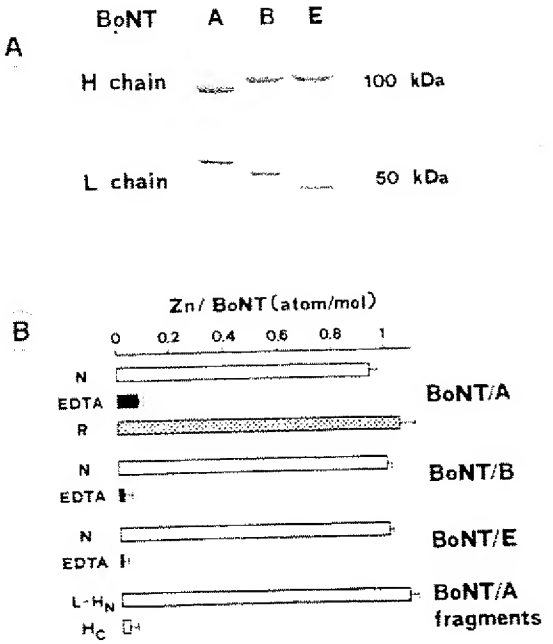

FIG. 2. **Zinc content of botulinum neurotoxins A, B, and E.** *A*, SDS-polyacrylamide gel electrophoresis and Coomassie Blue-stained samples of BoNT serotype A (*lane A*), B (*lane B*), and E (*lane E*). *B*, amounts of zinc bound to the dichain form of BoNT serotypes and to the two peptic fragments of BoNT/A, 100-kDa L-H_N, and 50-kDa H_C, before or after EDTA treatment, measured by atomic adsorption (details under "Materials and Methods"). *N*, native BoNT; *EDTA*, BoNT treated with EDTA and dialyzed; *R*, BoNT depleted of zinc with EDTA, incubated with 100 μM $ZnCl_2$, and dialyzed. *Bars* are ±S.D. of three different measurements.

of BoNT serotype A (the only L chain we have tested) was very low (not shown). This difference with the parent 150-kDa molecule indicates a partial alteration of the Zn^{2+} binding site of the L chain probably due to exposure to dithiothreitol and urea during its separation from H chain and isolation.

Zn^{2+} was also removed, with lower efficiency, by diethylenetriamine pentaacetic acid, a chelating agent specific for heavy metals (Arslan *et al.*, 1985). *O*-Phenantroline and dipicolinic acid, which are frequently used as heavy metal complexing agents, caused protein aggregation and an irreversible loss of zinc.

Zinc Coordination—The three-dimensional structures of three zinc metalloendopeptidases, thermolysin (Matthews *et al.*, 1972), *Bacillus cereus* neutral protease (Pauptit *et al.*, 1988), and *Pseudomonas aeruginosa* elastase (Thayer *et al.*, 1991), have been resolved by x-ray crystallography. In these enzymes, the Zn^{2+} atom is bound via a tetrahedral coordination with the two histidines of the motif HEXXH, while the glutamic residue binds a water molecule, which is the third Zn^{2+} ligand. The fourth ligand is a glutamic residue that has been identified in bacterial zinc metalloendopeptidase but whose position is still to be determined in matrix metalloendopeptidases (Vallee and Auld, 1990a, 1990b) and in the clostridial neurotoxins.

The role of histidines in Zn^{2+} binding in the botulinum neurotoxin was tested with chemical modification experiments. DEPC is a protein-modifying agent specific for histidines, which are converted to *N*-carbethoxyhistidines with a characteristic absorbance at 243 nm (Miles, 1977). Figure 3 shows that a different number of histidine residues was modified per molecule of the three toxins. This is related to the different histidine contents of the three BoNTs (13, 7, and 14 for serotypes A, B, and E, respectively) and to their accessibility to DEPC. However, for each neurotoxin, two additional histidines/BoNT molecule were modified when they were in their Zn^{2+}-depleted form. Parallelly, there was no modification of tyrosines as monitored at 278 nm.

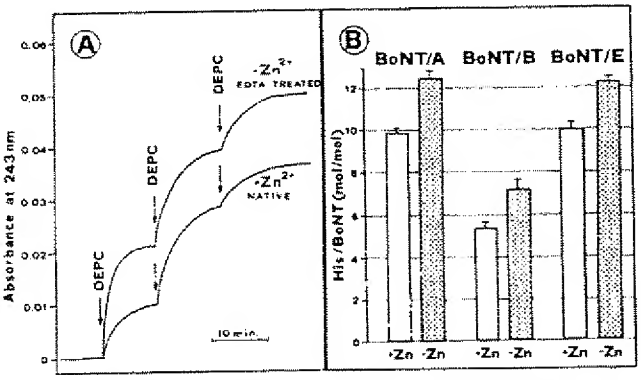

FIG. 3. **Histidine titration with DEPC of botulinum neurotoxin serotypes A, B, and E before and after Zn^{2+} depletion.** *A*, the increase of absorbance at 243 nm indicates the progressive formation of *N*-carbethoxyhistidine; successive addition of DEPC, necessary because of rapid DEPC decomposition in water, is indicated by *arrows*. *B*, amount of DEPC-modified histidines in the three BoNTs tested here before (*empty bars*) and after (*dotted bars*) Zn^{2+} removal. *Bars* are ±S.D. of at least three different experiments.

This result indicates that (i) the Zn^{2+} ion of BoNT protects two histidine residues from DEPC modification and (ii) these two histidines become accessible to DEPC when the zinc atom is absent. Each of the three BoNTs was unable to regain the Zn^{2+} atom after Zn^{2+} depletion and DEPC modification (see

below). It was not possible to test the effect of Zn^{2+} depletion on the neuroparalytic activity of BoNT because of the reversibility of Zn^{2+} depletion and because Zn^{2+}-undepleted and DEPC-modified BoNT is no longer toxic (not shown).

Cysteine residues coordinate Zn^{2+} in several proteins in which the zinc atom plays a structural role, such as in aspartate carbamoyltransferase, zinc finger proteins, and metallothioneins (Vallee and Auld, 1990a, 1990b). Titration of the sulfhydryl group of BoNT serotypes A, B, and E before and after Zn^{2+} depletion gave the same value (not shown), thus indicating that cysteines are not implicated in zinc binding in BoNTs.

These results are in agreement with the prediction, based on the sequence comparison of Fig. 1, that histidines, and not cysteines, are involved in Zn^{2+} coordination. Moreover, they suggest that the zinc ion of BoNT plays a catalytic role as in all zinc enzymes with histidine coordination (Vallee and Auld, 1990a, 1990b).

$[^{65}Zn]^{2+}$ Binding to BoNT and the Synthesized Peptide, Residues 226–240—To gain further evidence that the central segment of the BoNTs L chain is responsible for Zn^{2+} coordination, the peptide H_2N-Ile^{226}-Leu-Met-His-Glu-Leu-Ile-His-Val-Leu-His-Gly-Leu-Tyr-Gly^{240}-COOH, spanning the corresponding sequence of BoNT/B, which includes the putative zinc binding motif, was synthesized. Results of dot blot experiments (Fig. 4) show that this peptide binds $[^{65}Zn]^{2+}$ and suggest that the conserved histidine-rich segment of BoNTs and tetanus toxin can take part in zinc coordination. Fig. 4 also shows that both the zinc-depleted ($-Zn^{2+}$) and the native BoNT ($+Zn^{2+}$) serotypes A and E bind $[^{65}Zn]^{2+}$. This indicates that bound zinc is exchangeable. This exchange is not a peculiarity of the dot blot assay because it also occurred with BoNT in solution (Fig. 5).

BoNTs depleted of zinc and subsequently treated with DEPC were unable to bind $[^{65}Zn]^{2+}$ (Fig. 4), whereas a parallel DEPC treatment of the native BoNT did not affect its ability

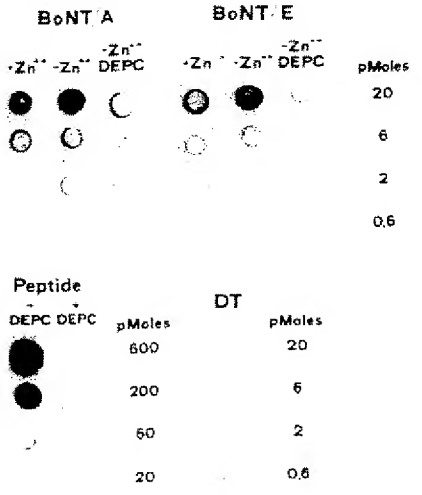

FIG. 4. **$[^{65}Zn]^{2+}$ binding to botulinum neurotoxins A and E and to the histidine-rich conserved peptide 226–240 of BoNT/B.** Protein (0.6–20 pmol) or peptide (20–600 pmol) samples, blotted onto nitrocellulose paper, were incubated in 50 nM $^{65}ZnCl_2$. After washings, the paper strips were dried and autoradiographed. Samples are as follows: *$+Zn^{++}$*, native BoNT; *$-Zn^{++}$*, BoNT treated with EDTA and dialyzed; *$-Zn^{++}$ DEPC*, BoNT treated with EDTA, dialyzed, and treated with DEPC; *peptide*, peptide 226–240 of BoNT/B not treated (−) or treated (+) with DEPC (details under "Materials and Methods"). Native BoNT treated with DEPC binds $[^{65}Zn]^{2+}$ as the untreated control ($+Zn^{++}$) (not shown). DT refers to samples of diphtheria toxin.

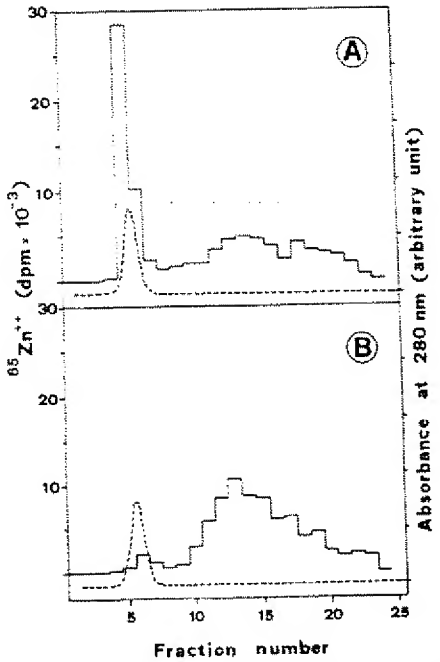

FIG. 5. **Zinc exchange on botulinum neurotoxin serotype E.** Elution of BoNT/E or diphtheria toxin from a Sephadex G-25 column after incubation of the toxins with 200 pM $^{65}ZnCl_2$ for 60 min at 25 °C. The *broken lines* show absorbance at 280 nm, and the *continuous lines* report the amount of radioactivity associated to the various fractions. *A*, BoNT serotype E; *B*, diphtheria toxin. Under the present conditions, 45% of added $[^{65}Zn]^{2+}$ coeluted with the BoNT peak, and practically none coeluted with the diphtheria toxin peak.

TABLE I

Flow dialysis and Scatchard analysis for $[^{65}Zn]^{2+}$ binding to BoNT/A, B, and E

Toxin	K_D	n	K_D	n
	nM		*μM*	
BoNT/A[a]	60–80	1–1.1	1.1–1.4	2.2–3.1
BoNT/B[b]	90–100	0.7–1.1	1.6–2.2	1.9–2.6
BoNT/E[b]	80–130	1	1.4–2.4	2.5–3.0

[a] Range of values found in three different experiments.
[b] Range of values found in two different experiments.

to exchange the Zn^{2+} atom (not shown). This result indicates that histidines are involved in zinc coordination in BoNT. The dot blot assay used here appears to be very specific since diphtheria toxin, which has three histidines interspaced by three residues (His^{484}-*X*-*X*-*X*-His-*X*-*X*-*X*-His^{492}), does not show any sign of $[^{65}Zn]^{2+}$ binding (Figs. 4 and 5).

Affinity of Zinc Binding to Botulinum Neurotoxins—The affinity of zinc binding to the three BoNTs was assayed by equilibrium dialysis, employing $[^{65}Zn]^{2+}$ as tracer (Colowick and Womack, 1969; Papini *et al.*, 1989). Table I shows that the three BoNT serotypes bind zinc very similarly. The three BoNTs have a single high affinity binding site (n close to 1) with comparable K_D values and also show multiple lower affinity sites that are not occupied in the purified toxins to account for the above described atomic adsorption data.

Conclusions—The present paper demonstrates that botulinum neurotoxins (serotypes A, B, and E) contain one atom of zinc/molecule of 150-kDa protein and that the Zn^{2+} atom can be reversibly removed with EDTA. This work also shows that histidines, and not cysteines, are involved in metal coordination, thus suggesting that the zinc atom plays a catalytic rather than a structural role (Vallee and Auld, 1990a, 1990b).

Comparison of amino acid sequences and $[^{65}Zn]^{2+}$ binding experiments suggests that the histidine-rich segment, conserved among clostridial neurotoxins, is involved in zinc coordination. This segment contains the zinc binding motif of metalloendopeptidases, including the glutamic residue directly involved in catalysis (Matthews, 1988). All of the three BoNTs assayed here show a single high affinity binding site for zinc with dissociation constants in the 40–100 nM range and multiple lower affinity binding sites.

Clostridia produce a variety of zinc endopeptidases (Bond and Van Wart, 1984), and, on the basis of our observations, it is tempting to speculate that clostridial neurotoxins have arisen by fusion of a gene coding for a metalloprotease with that of a protein highly specific for binding to the presynaptic membrane. The metalloprotease activity, confined in the L chain, could thus be delivered inside the neuronal cell and act on a specific peptide bond(s) of a component involved in the control of neurotransmitter release.

The possibility that botulinum neurotoxins are zinc endopeptidases readdresses the research aimed at the discovery of the molecular pathogenesis of botulinum. For example, several site-directed mutagenesis experiments can be designed and the product can be tested for activity in *Aplysia* neurons (Poulain, 1988, 1990, 1991) or in permeabilized PC12 cells (Lomneth *et al.*, 1991). Mutation of the glutamic residue of the motif is expected to delete the BoNT-induced inhibition of neuroexocytosis. Indeed, chemical modification of about two carboxyl groups of BoNT serotype A and E causes detoxification (Woody *et al.*, 1989). A lower or nonexistent activity is also expected for the mutants at the two histidines of the motif as well as at the third zinc ligand residue, which we propose to be either Glu^{262} of BoNT/A (Glu^{267} of BoNT/B and Glu^{251} of BoNT/E) or Glu^{351} of BoNT/A (Glu^{357} of BoNT/B and Glu^{336} of BoNT/E) on the basis of their conservation among all clostridial neurotoxins.

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